Morphological, Molecular, and Chromosomal Discrimination of Cryptic *Anopheles (Nyssorhynchus)* (Diptera: Culicidae) from South America

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J. Med. Entomol. 35(5): 830–838 (1998)

ABSTRACT Based on similarity of male genitalia, the malaria vector *Anopheles trinkae* Faran from the eastern Andean piedmont of Colombia, Ecuador, Peru, and Bolivia was determined by Peyton (1993) to be a junior synonym of *An. dunhami* Causey, then known from a single locality in Amazonian Brazil. Following an appraisal of molecular, chromosomal, and morphological characters, we conclude herein that the 2 taxa are specifically distinct and remove *An. trinkae* from synonymy with *An. dunhami*. Eggs of the 2 species are distinguished easily by the anterior crown, long floats, and closed deck that occur only in *An. trinkae*. The X chromosome of larval polytenes is divisible into R and L arms in *An. dunhami*, but not in *An. trinkae*. A phenogram based on banding pattern scores from 18 random amplified polymorphic DNA primers separated with 100% resolution *An. dunhami*, *An. trinkae*, *Anopheles nuneztovari* Gabaldón and *Anopheles darlingi* Root. In the ITS2 region of rDNA, 25% of base sites distinguished *An. trinkae* from *An. dunhami* and 21% from the related *An. nuneztovari*; males of these 3 species had accessory glands of significantly different sizes. Preliminary isoenzyme screening indicated that 3 of 11 loci were diagnostic for separating *An. trinkae* from *An. dunhami*. The results indicate that *An. dunhami* is related more closely to *An. nuneztovari* than to *An. trinkae* and illustrate the merits of a multidisciplinary approach to mosquito systematics.

KEY WORDS accessory glands, chromosomes, DNA, egg morphology, isomorphic species, isoenzymes

IDENTIFICATION OF CRYPTIC species of malaria vectors by standard morphological characters is known to be problematic and frequently unsatisfactory (White 1979). Within the Neotropical subgenus *Nyssorhynchus* of *Anopheles*, intraspecific variation of traits used for keys to adult females often exceeds interspecific variation (Gabaldón and Aguilera 1940, Kitzmiller et al. 1973). The limitations of morphological taxonomy for resolving species boundaries stimulated the advocacy of multidisciplinary approaches to mosquito systematics (Faran 1979a).

Anopheles (Nys.) trinkae was described by Faran (1979b), on the basis of morphological characteristics of larvae, pupae, and adult male genitalia of specimens collected in lowlands near the eastern slopes of the Andes, as specifically distinct from its presumed relatives *An. nuneztovari* Gabaldón and *An. rangeli* Gabaldón, Cova Garcia and Lopez. Owing to difficulties in separating these 3 taxa by adult female morphology, Faran (1979b) cautioned that some malaria transmission formerly attributed to *An. nuneztovari* in Colombia (Elliot 1968) or *An. rangeli* in Ecuador (Forattini 1962) might have been by *An. trinkae*. Subsequently, *An. trinkae* was incriminated as the primary vector of malaria attributable to *Plasmodium vivax* (Grassi and Feletti) among indigenous inhabitants of Junín Department, Peru (Hayes et al. 1987).

Based on morphological characteristics of specimens collected in Amazonian Brazil, *An. (Nys.) dunhami* was described by Causey (1945) and recognized as specifically distinct from *An. nuneztovari*. Although *An. dunhami* was captured commonly at animal baits at its type locality in Tefé, it was not recognized elsewhere in the Brazilian Amazon in the comprehensive collections of Deane et al. (1948). Lane (1953) regarded *An. dunhami* as a synonym of *An. nuneztovari*, and Faran (1980) accepted this opinion. Based on characters of the male genitalia, Peyton (1993) resurrected *An. dunhami* as a distinct species and sunk *An. trinkae* as its junior synonym. Recent regional keys for identifying anopheline species have followed Peyton (1993) and regarded *An. trinkae* as a synonym of *An. dunhami* (Calderón-Falero 1994).

In recent publications we also accepted Peyton's (1993) use of *An. dunhami* as a senior synonym of *An.*

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0022-2585/98/0830–0838$02.00/0

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1998		**00-00-1998 to 00-00-1998**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Morphological, Molecular, and Chromosomal Discrimination of Cryptic Anopheles(Nyssorhynchus) (Diptera: Culicidae) from South America	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Walter Reed Biosystematics Unit,Smithsonian Institution,Washington,DC,20560**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
see report

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**10**	

Table 1. Sources and methods of analyses of *An. dunhami* and *An. trinkae*

Collection site	Coordinates	Dates	Methods	No. specimens (families)[a]
An. dunhami				
Tefé, BR	3° 22′ N, 64° 43′ W	VIII-1994	MOL, CHR, MOR(A,P,L,E,ACC)	156 (6)
Tabatinga, BR	4° 13′ S, 69° 55′ W	VIII-1995	MOR(E)	1
An. trinkae				
Puerto Grether, Santa Cruz Dept., BO	17° 10′ S, 64° 20′ W	XI-1991	MOL	
Villa Tunari, Cochabamba Dept., BO	16° 53′ S, 65° 24′ W	XII-1992	MOL	
Chapare, Cochabamba Dept., BO		I-1982, 1983	MOR(A)	38
Carasco Tropical, Cochabamba Dept., BO		I-1995	MOR(A,P,L)	28 (5)
Coca, Napo Prov., EC	0° 28′ S, 76° 58′ W	VIII-1992	MOL	
Sardina Yacu, Napo Prov., EC	0° 5′ S, 77° 5′ W	VIII-1992	MOL, CHR, MOR(A,P,L,E,ACC)	141 (11)
Coca, Napo Prov., EC	0° 28′ S, 76° 58′ W	VIII-1992	MOL	
Lago Agrio, Sucumbios Prov., EC	0° 5′ N, 76° 53′ W	VIII-1992	MOL	
Puyo, Pastaza Prov., EC[b]		V-1977	MOR(A,P,L)	74
Villavicencio, Meta Dept., CO		1948–1949	MOR(A)	9
La Reforma, Meta Dept., CO		1964–1965	MOR(A)	1
Puerto Ocopa, Junin Dept., PE		V-1983	MOR(A)	1
Satipo, Junin Dept., PE		1985	MOR(A)	48

BR, Brazil; BO, Bolivia; CO, Colombia; EC, Ecuador; PE, Peru; MOL, molecular; CHR, chromosomes; MOR, morphology; A, adults; P, pupae; L, larvae; E, eggs; ACC, accessory glands.

[a] Refers only to specimens examined morphologically.

[b] Includes allotype and paratype male.

trinkae and applied the former epithet to refer to specimens from subandean Ecuador and Bolivia (Linley and Lounibos 1993, Lounibos 1994). However, after examining specimens collected in 1994 from the type locality of *An. dunhami* in Tefé, Brazil, we concluded that *An. dunhami* and *An. trinkae* should be regarded as separate species and noted this distinction in subsequent publications (Fritz et al. 1995, Conn et al. 1997, Lounibos et al. 1997). The purpose of the current article is to present multifaceted evidence that *An. trinkae* and *An. dunhami* are specifically distinct and to compare their relationships to the better-known *An. nuneztovari*.

Materials and Methods

Blood-fed females captured in Brazil, Ecuador, and Bolivia (Table 1) at human or animal baits yielded eggs of *An. dunhami* and *An. trinkae* that were distinguished from related species and one another based on the analyses described below. Portions of selected egg clutches were preserved for scanning electron microscopy (SEM) or link-reared to provide other life stages for morphological analyses or dissections. Other progeny from these broods were frozen at −70°C or preserved in 95% EtOH for subsequent molecular characterizations. Salivary glands were dissected from freshly killed F_1 4th instars according to the protocol of Conn (1990), and accessory glands were dissected from unmated, 2- to 4-d-old F_1 males as described in Lounibos (1994). Adult, larval, and pupal specimens borrowed from the Smithsonian Institution were examined for morphological characters (Table 1). Adults of *An. nuneztovari* and *An. darlingi* Root used for random amplified polymorphic DNA (RAPDs) comparisons were progeny of field collections from Peixoto de Azevedo, Mato Grosso State, Brazil. Sources of *An. nuneztovari* used for other comparisons are indicated elsewhere in the text, tables, or figure legends.

Link-rearing and preserving of larvae, pupae, and adults were performed as described in Belkin et al. (1965), and voucher specimens of these acquisitions have been deposited at the Walter Reed Biosystematics Unit of the Smithsonian Institution and at the Florida Medical Entomology Laboratory of the University of Florida. Adult wings were examined under polarized light to discriminate subtle scale colors, as recommended by Peyton and Ramalingam (1988). Eggs fixed in alcoholic Bouin's solution were prepared and examined with a Hitachi S-510 SEM according to methods in Linley and Lounibos (1993). Detailed descriptions of chorionic ultrastructure are provided elsewhere for *An. trinkae* (Linley and Lounibos 1993), *An. dunhami* (Lounibos et al. 1997), and *An. nuneztovari* (Linley et al. 1996). Lengths and widths of accessory glands dissected in saline were measured at 80x with an ocular micrometer, and gland volumes estimated by applying the formula for a cylinder (Lounibos 1994).

The DNA was extracted from previously frozen specimens of *An. dunhami*, *An. nuneztovari*, *An. trinkae*, and *An. darlingi* according to methods described in Fritz et al. (1994) and Wilkerson et al. (1993). Twenty decamer RAPD primers were selected from Operon primer sets A, B, and C (Operon, Alameda, CA). These primers were used to polymerase chain reaction (PCR)-amplify random fragments of the total DNA extract, after which the amplified fragments were separated on agarose minigels (Wilkerson et al. 1993, 1995). Eighteen of the 20 primers produced consistent scorable bands in at least 1 of the 4 species under consideration (Table 2; *Appendix 1*). The resultant data set was formatted as described in Black (1995), and a 1-S distance matrix was generated using the similarity option in the RAPDPLOT program

Table 2. Summary of RAPD primers, resultant fragment sizes, and corresponding columns in *Appendix No. 1*

Primer	Sequence	Band size, bp	Columns (*Appendix No. 1*)
OPC10	5′-TGTCTGGGTG-3′	3,024; 1,956; 1,537; 1,043	1-4
OPC06	5′-GAACGGACTC-3′	1,381; 954; 663; 544; 430	5-9
OPB17	5′-AGGGAACGAG-3′	1,254; 1,089; 1,013; 854	10-13
OPA07	5′-GAAACGGGTG-3′	1,212; 1,137; 955; 846; 779; 622; 561	14-20
OPA09	5′-GGGTAACGCC-3′	1,410; 1,137; 1,004; 829; 394	21-25
OPB04	5′-GGACTGGAGT-3′	1,187; 1,078; 766; 507; 470; 455	26-31
OPB12	5′-CCTTGACGCA-3′	1,921; 1,535; 962; 748; 609	32-36
OPA20	5′-GTTGCGATCC-3′	1,021; 785; 770; 382	37-40
OPB03	5′-CATCCCCCTG-3′	2,431; 1,918; 1,524; 1,278; 959; 373	41-46
OPB15	5′-GGAGGGTGTT-3′	1,948; 1,478; 1,377; 1,068; 582; 374	47-52
OPB05	5′-TGCGCCCTTC-3′	1,423; 1,182; 986; 872; 855; 823; 791; 534	53-60
OPB08	5′-GTCCACACGG-3′	1,454; 1,423; 1,336; 758; 676; 727; 536; 476; 321	61-69
OPA18	5′-AGGTGACCGT-3′	1,337; 775	70-71
OPA05	5′-AGGGGTCTTG-3′	1,462; 1,306; 1,264; 962; 796; 767; 716	72-78
OPC18	5′-TGAGTGGGTG-3′	1,729; 1,548; 1,294; 1,154; 754; 701	79-84
OPB01	5′-GTTTCGCTCC-3′	1,725; 1,570; 1,482; 1,033; 704; 612; 573; 482; 357	85-93
OPA04	5′-AATCGGGCTG-3′	1,288; 1,020; 955; 714; 603; 354	94-99
OPA08	5′-GTGACGTAGG-3′	1,753; 1,478; 1,399; 996; 939; 725; 621; 230	100-107

(Black 1995). The formula is derived from the Nei and Li (1985) similarity index: $S = 2N_{AB}/(N_A + N_B)$ where N_{AB} are the fragments that 2 individuals share in common, and N_A and N_B are the number of fragments in individuals A and B, respectively. The matrix was analyzed in PHYLIP 3.5C using the NEIGHBOR program by the unweighted pair-group method with arithmetic mean average option, and a phenogram was produced with DRAWGRAM, also in PHYLIP 3.5C (Felsenstein 1993). RAPDBOOT (West and Black 1998) was used to generate 100 pseudoreplicate distance matrices, which were collapsed to form 100 trees with the unweighted pair-group method with arithmetic mean average. The bootstrap consensus tree was derived from the 100 the unweighted pair-group method with arithmetic mean average trees with the CONSENSUS program in PHYLIP 3.5C

The ITS2 region of rDNA of 4 individuals of *An. dunhami* from 1 collection site and 7 individuals of *An. trinkae* from 4 sites was amplified by PCR and sequenced according to instructions for Perkins-Elmer Applied Biosystems DNA kits (J. A. Danoff-Burg and J.E.C., unpublished data). The ITS2 sequence of *An. nuneztovari* is the consensus sequence derived from 10 collections of this species in 5 countries (Fritz et al. 1994).

Starch gel electrophoresis was performed on 3 individuals of *An. dunhami by* using protocol established for analyses of the isoenzymes *Hk-1, Pgi, Gdh, Mdh, Me, Fum, Had, Idh-1, Pgm, Adk-1,* and *Aat-1* of *An. trinkae, An. nuneztovari,* and *An. rangeli* (Fritz et al. 1995), whose allele frequencies were compared with those of *An. dunhami.* Polytene chromosomes were observed and photographed after squashing salivary glands of 4th instars and staining with aceto-lactic-orcein according to methods in Conn (1990).

Results

Biogeography. To date, *An. dunhami* has been identified only from its type locality in Tefé and from a recent collection at Tabatinga, Brazil (Lounibos et al. 1997), both sites are located on the Rio Solimões (Fig. 1). By contrast, the known range of *An. trinkae* now extends into Peru and Bolivia as a consequence of collections that followed Faran's (1979b) original description based on specimens from Colombia and Ecuador (Table 1). All records of *An. trinkae* come from lowland areas in close proximity to the eastern slopes of the Andes ranges (Fig. 1).

Morphology. Examinations of prepared slides confirmed Peyton's (1993) observation that male genitalia of *An. dunhami* and *An. trinkae* are indistinguishable. Pale wing spots of female *An. dunhami* ($n = 25$) were cream to tan, whereas the homologous spots of female *An. trinkae* ($n = 25$) were white to cream. Seta 9-VIII of the pupa was longer in all *An. trinkae* examined (mean segment length/setal length = 4.4 for *An. dunhami,* range = 4.2–4.7; mean = 2.6 for *An. trinkae,* range = 2.4–2.8; $n = 4$ for each species).

The most prominent morphological differences between *An. dunhami* and *An. trinkae* were observed in the eggs (Fig. 2). All *An. trinkae* eggs possessed an

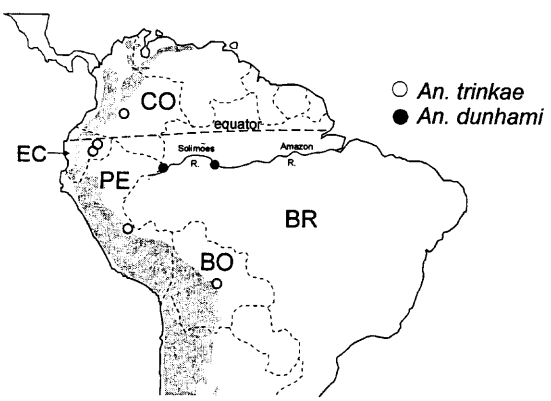

Fig. 1. Major collection localities of this study for *An. trinkae* and *An. dunhami* in South America. Shaded area represents the Andes mountains. Country abbreviations are in Table 1.

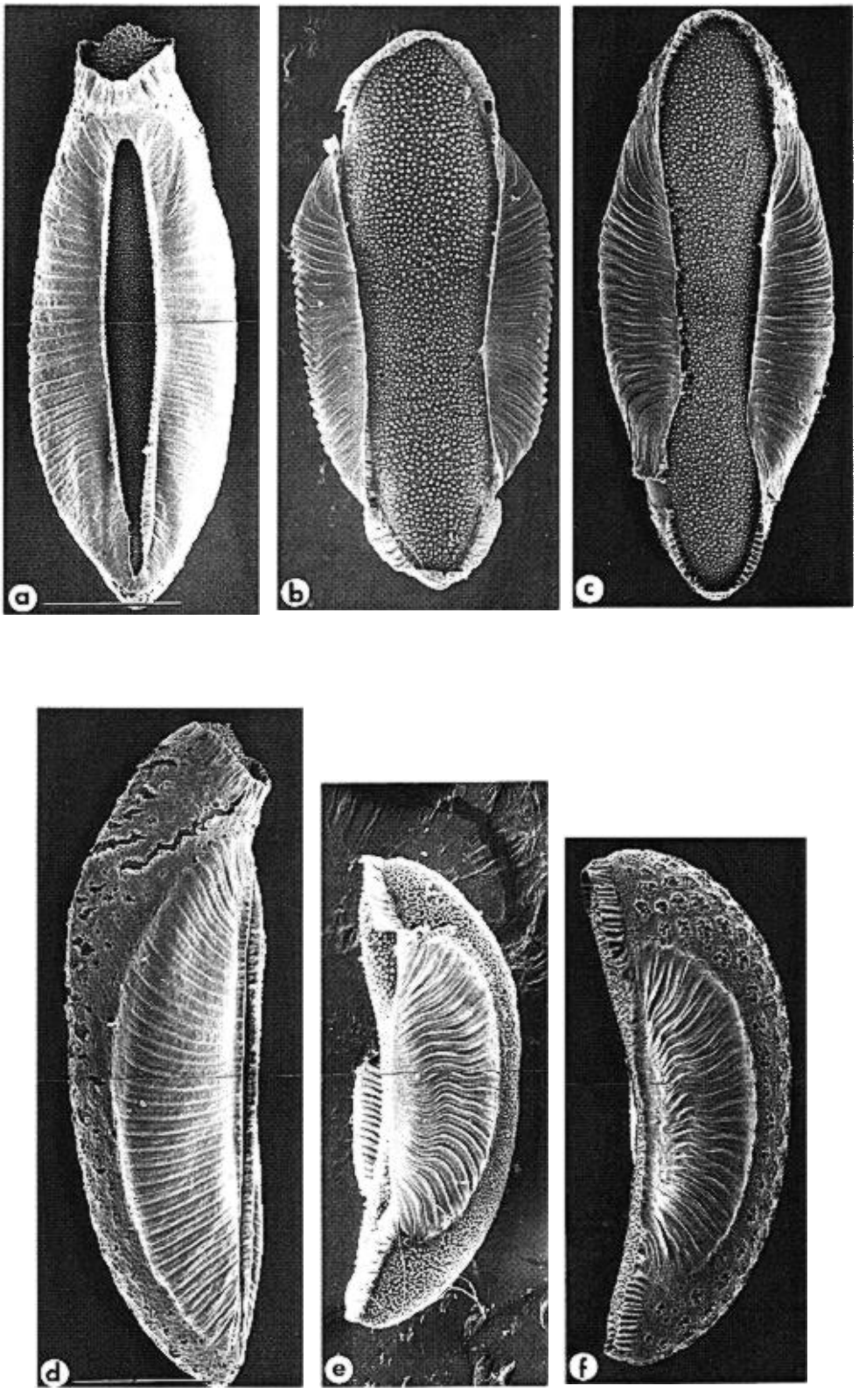

Fig. 2. Ventral (a–c) and lateral (d–f) views of eggs of *An. trinkae* (a and d) from Sardina Yacu, Ecuador; *An. dunhami* (b and e) from Tefé, Brazil, and *An. nuneztovari* (c and f) from Manaus, Brazil. Scale bar = 200 μm.

Table 3. Comparison of mean accessory gland volumes of 3 *Nyssorhynchus* species

Species	Site/country	No.[a]	Vol[b] ($\times 10^{-2} mm^3$) (SE)
An. nuneztovari	Belém BR	14	1.60A (0.07)
An. nuneztovari	Manaus BR	13	1.63A (0.08)
An. nuneztovari	Porto Velho BR	10	1.34A (0.08)
An. dunhami	Tefé BR	11	0.54B (0.08)
An. trinkae	Sardina Yacu EC	32	0.10C (0.05)

[a] Laboratory-raised male progeny of field-collected females.
[b] Means adjusted after analysis of covariance with wing length as covariate are significantly different ($P < 0.05$) if followed by different letters after testing by a Ryan-Einot-Gabriel-Welsch multiple comparisons test with PROC GLM of SAS Institute (1985).

anterior crown and a narrow deck region enclosed by ventrally positioned floats (Fig. 2 a and d), whereas *An. dunhami* had an exposed deck region flanked by smaller, more laterally positioned floats and open anterior and posterior frills (Fig. 2 b and e). The *An. dunhami* egg resembles more closely that of *An. nuneztovari,* but is distinguished from the latter by the absence of the raised, pore-ridden mounds of the dorsal plastron that are common to all *An. nuneztovari* (Fig. 2 e *compare* f, Linley et al. 1996). The ventral deck region usually is more exposed in *An. dunhami* than in *An. nuneztovari* (Fig. 2 b *compare* c).

The adjusted mean volume of male accessory glands from *An. dunhami* was slightly >5-fold the volume of the same glands from *An. trinkae* (Table 3). Both species had significantly smaller accessory glands than *An. nuneztovari,* whose mean gland volumes did not differ significantly among 3 geographic samples from Brazil (Table 3, Lounibos 1994).

Molecular Characterizations. The 18 scorable primers used in this study produced 107 scorable bands ranging from ≈3.024 to 0.230 kbp (Table 2). Individuals clustered into 4 groups corresponding to their presumptive species (Fig. 3). Branch lengths within clusters were small in comparison to lengths among clusters. All 4 clusters were supported by bootstrap values of 100. Although not supported strongly by genetic distance and bootstrap values, *An. dunhami* and *An. nuneztovari* were more similar to each other than they were to *An. trinkae* and *An. darlingi.*

Anopheles dunhami and *An. trinkae* differed at 25% of nucleotide sites of the ITS2 region, and *An. nuneztovari* and *An. trinkae* differed at 21% (Fig. 4). By contrast, *An. dunhami* and *An. nuneztovari* differed in 6% of base pairs of this same region of rDNA. Intraspecific variation in ITS2 nucleotide sites was 0.0% for *An. dunhami* ($n = 4$), 1.7% for *An. trinkae* ($n = 7$), and 1.1% for *An. nuneztovari* ($n = 10$) (J. A. Danoff-Burg and J.E.C., unpublished data).

Three of 11 isoenzyme loci were diagnostic for separating *An. trinkae* from *An. dunhami,* which was homozygous at *Hk-1* (rf = 102), *Pgi* (rf = 96) and *Gdh* (rf = 76). *An. trinkae* does not have an allele in common with *An. dunhami* at these loci (Fritz et al. 1995; *Hk-1,* rf = 117; *Pgi,* rfs = 108,100; *Gdh,* rfs = 95,100). Of 11 loci tested, only *Gdh* diagnosed *An. dunhami*

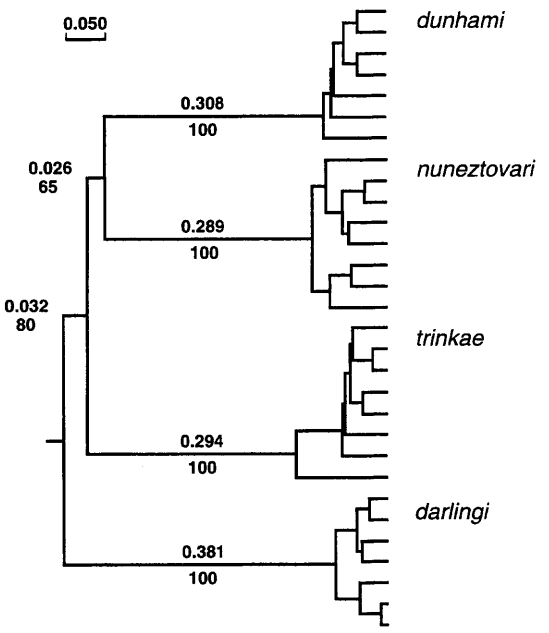

Fig. 3. Phenogram derived from RAPD fragment analyses showing relationships among individuals belonging to 4 species of *Anopheles (Nyssorhynchus).* Branch lengths are proportional to 1-S, where S is the similarity index defined in the text. The top number on selected branches is 1-S phenetic distance and the bottom the number of bootstrap replicates out of 100 supporting the topology.

from 2 Venezuelan populations of *An. nuneztovari* (Fritz et al. 1995).

Chromosomes. The X chromosome of salivary polytenes of *An. dunhami* from Tefé ($n = 5$, progeny of 3 females) was divisible into XR and XL arms. By contrast, the X chromosome of all *An. trinkae* from Ecuador ($n = 8$, progeny of 5 females) was telocentric (i.e., not divisible into XR and XL arms). Progeny of each of the 2nd group of families were confirmed as *An. trinkae* by the mtDNA profiles described in Conn et al. (1997).

Discussion

Our study demonstrated the merits of investigating related taxa across all levels of organization (i.e., from the molecular to the organismic) (Wilson 1989). This approach to mosquito systematics required the multiple use of field-collected specimens that exceeded the traditional scope of museum-based taxonomy.

Peyton (1993) concluded that *An. trinkae* and *An. dunhami* were conspecific based on their indistinguishable male genitalia. Isomorphic male genitalia are common among members of anopheline species complexes (e.g., *Anopheles gambiae* Giles [Gillies and DeMeillon 1968] and *Anopheles culicifacies* Giles [Harrison 1980]). However, in contrast to the genetic affinities of sibling members of species complexes, *An. dunhami* is related more closely to *An. nuneztovari*

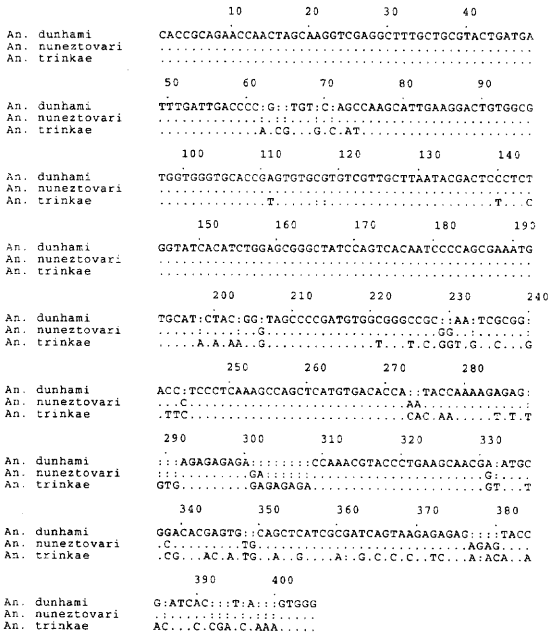

Fig. 4. Sequence alignment of the ITS2 region of rDNA for *An. trinkae* (consensus sequence from 4 sites), *An. dunhami* (Tefé, Brazil) and *An. nuneztovari* (consensus sequence from 10 sites in Fritz et al. [1994]). GenBank accession numbers are U92346 and U92355 (*An. trinkae*); U92326 (*An. dunhami*); U92343, U92350, and U92351 (*An. nuneztovari*).

than to *An. trinkae*, and we therefore speculate that the isomorphic male genitalia may have arisen by convergent evolution.

The egg stage offered the most obvious morphological structures for separating *An. trinkae* from *An. dunhami* (Fig. 2), as it has for identifying other cryptic species of *Anopheles* since Falleroni (1926) recognized members of the *An. maculipennis* complex from egg structures. Our SEM examinations corroborated the description of the *An. dunhami* egg based on the light microscope observations of Causey (1945) and resolved much more morphological detail, as depicted in Lounibos et al. (1997). Characters that separate *An. dunhami* and *An. trinkae*, such as the presence or absence of the anterior crown, or size and position of the floats, can be distinguished readily under a dissecting microscope. In egg morphology, *An. trinkae* and *An. rangeli* are relatively similar to each other (Linley and Lounibos 1993), as are *An. dunhami* and *An. nuneztovari*. The length of pupal seta 9-VIII also may diagnose *An. dunhami* from *An. trinkae*, but this character warrants validation with larger sample sizes.

Currently available collection records of the 2 species indicate no overlap in their geographic distributions, although it is possible that female specimens from eastern Peru and western Brazil may have been misidentified by collectors as *An. rangeli* or *An. nuneztovari*. From comprehensive collections throughout the Brazilian Amazon in the 1940s, Deane et al. (1948) recorded *An. dunhami* only from Tefé. The Tefé area is known for its endemic plants (Prance 1987) and butterflies (Brown 1987) and also may have provided a refugium for the isolation and divergence of *An. dunhami* from *An. nuneztovari*.

The 2 species also differ in their importance as malaria vectors. *An. trinkae* was incriminated as a vector of human malaria in eastern Peru (Hayes et al. 1987) and commonly bites humans in eastern Ecuador and central Bolivia where malaria is endemic but vector incriminations remain incomplete (L.P.L., L.J.H., J.E.C., and G.N.F., unpublished data). *An. darlingi* does not occur in such subandean localities but is the primary vector in Tefé, Brazil, where *An. dunhami* was captured only in rural areas of relatively low human density (L.P.L. and J.E.C., unpublished data). In Amazonian Peru where *An. darlingi* is currently the vector of epidemic malaria, wild-caught females identified morphologically as *An. nuneztovari* have tested positive for human malaria in sporozoite enzyme immunoassays (Fernandez et al. 1997). In this zone of possible geographic overlap of *An. nuneztovari* with *An. dunhami* and *An. trinkae*, identifications should be corroborated with one or more of the methods used in the current study to separate cryptic species.

Between sibling species of *Anopheles*, base pair differences in the ITS2 regions of rDNA may either be common or rare. For example, 18.5–28.7% of base sites separated species pairs of the 5 members of the *An. quadrimaculatus* complex (Cornel et al. 1996), but 5 species of the *An. gambiae* complex differed by only 0.4–1.6% in this same region (Paskewitz et al. 1993). Given the low intraspecific variability observed for the ITS2 of *An. dunhami* (0.0% for $n = 4$) and *An. trinkae* (1.7% for $n = 7$), we conclude that the 25% interspecific difference in ITS2 nucleotide sites demonstrates substantial genetic divergence between these 2 species. The RAPD data also support the existence of *An. trinkae* as a genetic entity distinct from *An. dunhami* and corroborate other evidence that *An. dunhami* is phenetically closer to *An. nuneztovari* than to either *An. darlingi* or *An. trinkae*.

Although our isoenzyme results should be interpreted cautiously because only 3 *An. dunhami* were screened for 11 loci, 3 of these (27.3%) were diagnostic for separating this species from *An. trinkae*. By contrast, 3 of 24 (12.5%) loci were diagnostic for distinguishing *An. trinkae* from 8 geographic populations of *An. rangeli* (Fritz et al. 1995). These preliminary results indicate that *An. trinkae* may be related more closely to *An. rangeli* than to *An. dunhami*.

Species of *Nyssorhynchus* are separable by their X chromsomes (Kitzmiller et al. 1973, Kitzmiller 1977). Variation in the position of the centromere determines whether the X has L and R arms, as in *An. dunhami*, or is telocentric owing to a terminal centromere, as in *An. trinkae*. With respect to gross morphology of the X chromosome, *An. dunhami* would appear to be more similar to *An. nuneztovari* which has L and R arms (Kitzmiller et al. 1973, Conn 1990).

In conclusion, *An. trinkae* is a valid species that we herein resurrect from synonymy from *An. dunhami*. The latter species is related more closely to *An. nun-*

eztovari. *An. trinkae* probably is related more closely to *An. rangeli*. All 3 conclusions are further supported by a recent, revised phylogeny of the subgenus *Nyssorhynchus* that synthesizes morphological and molecular characters (J.A. Danoff-Burg and J.E.C., unpublished data).

Acknowledgments

We are grateful to our many collaborators from South America whose cooperation made possible the collections of specimens used for this study. Especially important were R. Lourenço-de-Oliveira (Brazil), J. Alarcón (Ecuador), and H. Bermudez and R. Rodriguez (Bolivia). We thank J. Pecor for loan of specimens from the Walter Reed Biosystematics Unit of the Smithsonian Institution to the Florida Medical Entomology Laboratory, and W. Black for advice on RAPDs analysis and a critique of an earlier draft of this paper. Micrographs of eggs kindly were provided by D. Duzak and the late J. Linley. Research was supported by NIH grants AI-31034 and AI-40116. This is Florida Agricultural Experiment Station Journal Series No. R-06256.

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Received for publication 29 December 1997; accepted 28 April 1998.

Appendix 1. RAPD fragment data matrix for 4 species of *Anopheles (Nyssorhynchus)*. Individual specimen numbers correspond to terminal branches read from top to bottom on the phenogram in Fig. 3. See Table 2 for a summary of the primers used, the fragments produced, and the column to which each fragment corresponds

Column no.	10	20	30	40	50	60	70	80	90	100	
An. dunhami 1	0001110100	0000001000	0011000010	1110100010	1100000100	0001001000	0000100000	1000001000	0000001010	0000010000	1010000
An. dunhami 2	0001111100	0000001000	0011000000	1110100010	1100000100	0001001000	0000100000	1000001000	0000001000	0000110000	1010000
An. dunhami 3	0001101100	0000001001	0011000010	1110100000	0100000100	0001001000	0000100000	1000001000	0000001010	0000110000	0010000
An. dunhami 4	0001101100	0000001000	0011000010	1110100010	1100000100	0001001000	0000100000	1000001000	0000001010	0000110000	0010001
An. dunhami 5	0001100100	0000001001	0011000010	1110101000	1100000100	0001001000	1000100000	1000001000	0100001010	0000110000	1010001
An. dunhami 6	0001101100	0000001000	0011000010	1110100000	0000000100	0001001000	0000100000	1000001000	0100001001	0000110000	1010000
An. dunhami 7	0001111100	0000001000	0001000010	1110100010	0000000100	0000001000	0000100000	1000001000	0000001010	0000100000	1010000
An. trinkae 1	1110100001	1000110101	0000001000	1010010011	0011000110	1000001000	1011001000	0110001011	0100110100	0010001101	0100100
An. trinkae 2	1110100001	1000110101	0000001000	1010010011	0001101010	1000001010	1001000100	0110000011	0100110100	0010001101	0100100
An. trinkae 3	1110100001	1000110101	0000001000	1010010011	0001101010	1000001000	1001000000	0110001011	0100110100	0010001101	0100100
An. trinkae 4	1110000001	0000110101	0000001000	1010010011	0011001010	1000001010	1001001000	0110001010	0100110100	0010001101	0100100
An. trinkae 5	1110000001	1000100101	0000001000	1010010001	0001001010	1000001010	1001001100	0110001010	0100110100	0010001101	0100100
An. trinkae 6	1110000001	1000110101	0000001000	1010010011	0011001100	0000001000	1001000100	0110001011	0100110100	0010001101	0100000
An. trinkae 7	1110000001	0000110101	0000001000	1010010001	0001100000	1000001000	1001001000	0110001011	0000110100	0010001101	0100100
An. trinkae 8	1110000001	1000110000	0000000000	1010000001	0001100000	1000001000	1001000101	0111001010	0000110100	0000000100	0100100
An. darlingi 1	0000000000	0111000010	1101101100	1011001000	0000100001	0010010101	0000010010	0000010100	1010000000	0100000010	0011010
An. darlingi 2	0000000000	0111000000	1101100100	1011001000	0000100001	0010010101	0000010010	0000010100	1010000001	0100000010	0011010
An. darlingi 3	0000000000	0111000010	1101100100	1011001000	0000100001	0010000100	0000010010	0000110100	1010000001	0100000010	0011010
An. darlingi 4	0000000000	0111000000	0101100100	1011001000	0000100001	0010000100	0000010010	0000010100	1010000001	0100000010	0010010
An. darlingi 5	0000000000	0111000010	1101101101	1010001000	0000010001	0010000100	0000010010	0000110000	1010000000	0100000010	0011010
An. darlingi 6	0000000000	0111000000	1101100101	1011001000	0000010001	0010000100	0000010010	0000110000	1010000000	0100000010	0001010
An. darlingi 7	0000000000	0111000000	1101100101	1011001000	0000010001	0010000100	0000010010	0000110000	1010000000	0100000010	0011010
An. nuneztovari 1	0000100011	0000001001	0000010011	0001100010	0000000000	0100101011	0110001001	0011000000	0001000000	0001000000	0000001
An. nuneztovari 2	0000100011	1000000011	0000010011	0001100100	0000000000	0100101001	0110000001	0011001000	0001000000	1001000000	0000001
An. nuneztovari 3	0000100011	1000001011	0000010011	0001100100	0000000000	0100101001	0110000001	0001000000	0001000000	1001000000	0000001
An. nuneztovari 4	0000100011	0000001010	0000010011	0001100100	0000000000	0100101001	0110000001	0010001000	1001000000	1001000000	0000001
An. nuneztovari 5	0000100011	0000001010	0000010011	0000100100	0000000000	0100101000	0110000001	0011001000	0000000000	1001000000	0000001
An. nuneztovari 6	0000100010	0000001000	0000000011	0001100100	0000000000	0100101000	0110001001	0011000000	0001000000	1001000000	0000001
An. nuneztovari 7	0000100010	0000000000	0000010011	0001100100	0000000000	0100001000	0110001001	0011000000	0000000000	1001000000	0000001
An. nuneztovari 8	0000100010	0000000010	0000010011	0001000100	0000000000	0000001000	0110001001	0011000000	0001000000	0001000000	0000001